FORM PTO-1390 (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER: IVD 1087

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/831720

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/FR99/02761	10 November 1999	17 November 1998

TITLE OF INVENTION:

USE OF A SUBSTANCE BINDING WITH THE PERIPHERAL BENZODIAZEPIN RECEPTOR FOR TREATING SKIN STRESS

APPLICANT(S) FOR DO/EO/US

CASELLAS, Pierre and DEROCQ, Jean-Marie

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND or SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1)).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☒ has been transmitted by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ A translation of the International Application into English (35 U.S.C. 371 (c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   b. ☐ have been transmitted by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3)).
9. ☒ An executed oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.

    ☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
    Citation of References

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO	ATTORNEY'S DOCKET NUMBER
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17. ☒ The following fees are submitted:

	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1. 492 (a)(1)-(5)):		
Search Report has been prepared by the EPO or JPO. $860.00		
International preliminary examination fee paid to USPTO (37CFR 1.482) $690.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) $710.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO $1000.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4). $100.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ]20 [ ]30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	26 - 20 =	6	x $18.00	$	108.00
Independent claims	5 - 3 =	2	x $80.00	$	160.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ $270.00	$	
TOTAL OF ABOVE CALCULATIONS =				$	1128.00
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				$	
SUBTOTAL =				$	1128.00
Processing fee of **$130.00** for furnishing the English translation later than [ ]20[ ]30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				$	
TOTAL NATIONAL FEE =				$	1128.00
Fee for recording the enclosed assignment (37 CFR 1.21(h). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	40.00
TOTAL FEES ENCLOSED =				$	1168.00
				Amount to be refunded:	$
				Charged	$ 1168.00

a. ☐ A check in the amount of $_____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. **19-0091** in the amount of **$1168.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **19-0091**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Paul E. Dupont
Patent Department
Sanofi-Synthelabo Inc.
9 Great Valley Parkway
P.O. Box 3026
Malvern, PA 19355

27546
PATENT TRADEMARK OFFICE

SIGNATURE 5/14/01 DATE

Paul E. Dupont
NAME

27,438
REGISTRATION NUMBER

(610) 889-6338
TELEPHONE NUMBER

Attorney Docket No. IVD 1087

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Filing under 35 U.S.C. § 371
Corresponding to International
Application Serial No.: PCT/FR99/02761

Applicants: CASELLAS, Pierre and
DEROCQ, Jean-Marie

International Filing Date: 10 November 1999

For: USE OF A SUBSTANCE BINDING WITH THE PERIPHERAL BENZODIAZEPIN RECEPTOR FOR TREATING SKIN STRESS

CERTIFICATE UNDER 37 C.F.R. 1.10

Express Mail Label Number: EL676470990US

Date of Deposit: May 14, 2001

I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" Service on the date indicated above and is addressed to: Commissioner for Patents, Box PCT, Attn: EO/US, Washington, DC 20231.

Paula S. Ackley

Signature

Commissioner for Patents
Box PCT
Attn: EO/US
Washington, D.C. 20231

Dear Sir:

PRELIMINARY AMENDMENT

Please amend the above-identified application as follows:

In the Specification:

At page 2, please replace the paragraph beginning at line 12 with the following rewritten paragraph.

--Many PBR ligands are disclosed in the literature (Figure 7). Examples which may be mentioned include Ro 5-4864 or chlorodiazepam, Ro 5-2807 or diazepam and PK 11195, or reference may be made to the article Peripheral Benzodiazepine Receptors, Ch. III, J.J. Bourguignon, Ed. E. Giesen - Crouse, Academic Press.--

Following the claims, add new page 29 containing the following Abstract of the disclosure.

--Abstract of the Disclosure

A composition containing a peripheral benzodiazepine receptor ligand for topical use in the treatment of cutaneous stress.--

In the Claims:

Please cancel claims 1-15 without prejudice to the prosecution of said claims in a continuing application.

Please add following new claims 16-41.

-- 16. (New) A topical composition for treating cutaneous stress containing as active principle a substance that binds to the peripheral benzodiazepine receptors.

17. (New) A composition according to Claim 16 wherein the substance that binds to the peripheral benzodiazepine receptor is a peripheral benzodiazepine receptor agonist chosen from synthetic molecules, natural extraction substances and substances obtained by fermentation.

18. (New) A composition according to Claim 17 wherein the substance that binds to the peripheral benzodiazepine receptor is RO 5-4864.

19. (New) A composition according to Claim 17 wherein the substance that binds to the peripheral benzodiazepine receptor is obtained by fermentation.

20. (New) A composition according to Claim 19 wherein the substance that binds to the peripheral benzodiazepine receptor is a fermentation product of *Nocardia* SRL 4988, *Streptomyces* SRL 5186 or *Actinosinnema* SRL 5189.

21. (New) A composition according to Claim 17 wherein the substance that binds to the peripheral benzodiazepine receptor is present in an amount of from 0.00001% to 20% by weight relative to the total weight of the composition.

22. (New) A composition according to Claim 21 wherein the substance that binds to the peripheral benzodiazepine receptor is present in an amount of from 0.001% to 10% by weight relative to the total weight of the composition.

23. (New) A composition according to Claim 17 additionally containing a hydroxy acid or a retinoid.

24. (New) A composition according to Claim 23 wherein the hydroxy acid is chosen from α-hydroxy acids and β-hydroxy acids which may be linear, branched or cyclic, saturated or unsaturated.

25. (New) A composition according to Claim 23 wherein the retinoid is chosen from retinoic acid and derivatives thereof, and retinol and esters thereof.

26. (New) A method for the treatment of cutaneous stress which comprises topically administering to a subject in need of such treatment an effective amount of a substance that binds to the peripheral benzodiazepine receptor.

27. (New) A method for the treatment of cutaneous stress which comprises topically administering to a subject in need of such treatment an effective amount of a composition according to Claim 17.

28. (New) A method for the treatment of cutaneous stress which comprises topically administering to a subject in need of such treatment an effective amount of a composition according to Claim 18.

29. (New) A method for the treatment of cutaneous stress which comprises topically administering to a subject in need of such treatment an effective amount of a composition according to Claim 19.

30. (New) A method for the treatment of cutaneous stress which comprises topically administering to a subject in need of such treatment an effective amount of a composition according to Claim 20.

31. (New) A method for the treatment of cutaneous stress which comprises topically administering to a subject in need of such treatment an effective amount of a composition according to Claim 21.

32. (New) A method for the treatment of cutaneous stress which comprises topically administering to a subject in need of such treatment an effective amount of a composition according to Claim 22.

33. (New) A method for the treatment of cutaneous stress which comprises topically administering to a subject in need of such treatment an effective amount of a composition according to Claim 23.

34. (New) A method for the treatment of cutaneous stress which comprises topically administering to a subject in need of such treatment an effective amount of a composition according to Claim 24.

35. (New) A method for the treatment of cutaneous stress which comprises topically administering to a subject in need of such treatment an effective amount of a composition according to Claim 25.

36. (New) A method for reducing wrinkles, reducing solar erythema or protecting against free radicals which comprises topically administering to a subject in need of such treatment an effective amount of a composition according to Claim 17.

37. (New) A method for reducing wrinkles, reducing solar erythema or protecting against free radicals which comprises topically administering an effective amount of a composition according to Claim 18.

38. (New) A method for reducing wrinkles, reducing solar erythema or protecting against free radicals which comprises topically administering an effective amount of a composition according to Claim 20.

39. (New) Strain *Nocardia species* SRL 4988 filed at the C.N.C.M. of the Institut Pasteur under No. I-2305 and its productive mutants.

40. (New) Strain *Streptomyces species* SRL 5186 filed at the C.N.C.M. of the Institut Pasteur under No. I-2306 and its productive mutants.

41. (New) Strain *Actinosinnema species* SRL 5189 filed at the C.N.C.M. of the Institut Pasteur under No. I-2307 and its productive mutants.--

REMARKS

The specification is amended at page 2, line 13 by inserting a reference to Figure 7 and to add an abstract following the claims.

Original claims 1-15 have been canceled without prejudice.

New composition claim 16 corresponds essentially to original claim 11 and new claims 17-25 depend from and further limit the composition of claim 11 in terms of the nature or concentration of its ingredients. The limitation of claims 17-19, and 21-25 correspond to the limitations of the compositions prepared according to original claims 3-5 and 6-10 respectively, and new claim 20 corresponds to original claim 15.

New claims 26, 27-29 and 31-35 correspond to original so-called Swiss-type "second indication" claims 1, 3-5 and 6-10 respectively, but written in appropriate U.S. method of treatment format. New claim 30 is directed to the method of using the composition of original claim 15.

New claims 36 and 37 correspond to original claim 3 and 4 insofar as the latter claims depend from claim 2. New claim 38 further defines the cutaneous stress of prior claim 30 as defined in original claim 2. New claims 39-41 correspond to original claims 12-14

No new matter is added by the amendment of the specification or the addition of new claims 16-41.

Attached hereto is a page entitled "Version With Markings To Show Changes Made" which is a marked-up version of the changes made to the specification and claims by the instant amendment.

Respectfully submitted,

Date: May 14 2001

Paul E. Dupont
Reg. No. 27,438

Address:
Patent Department
Sanofi-Synthelabo Inc.
9 Great Valley Parkway
Malvern, PA 19355
Telephone No. (610) 889-6338
Facsimile: (610) 889-8799

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In The Specification:

The paragraph beginning at page 2, line 12 has been amended as follows:

Many PBR ligands are disclosed in the literature (Figure 7). Examples which may be mentioned include Ro 5-4864 or chlorodiazepam, Ro 5-2807 or diazepam and PK 11195, or reference may be made to the article Peripheral Benzodiazepine Receptors, Ch. III, J.J. Bourguignon, Ed. E. Giesen - Crouse, Academic Press.

A new section entitled "Abstract of the Disclosure" has been added at new page 29 immediately following the claims.

In The Claims:

Claims 1-15 have been canceled and new claims 16-41 have been added.

ENGLISH TRANSLATION OF INTERNATIONAL PATENT APPLICATION PCT/FR99/02761 filed on 10 November 1999

CERTIFICATE UNDER 37 C.F.R. 1.10

Express Mail Label No.: EL676470990US

Date of Deposit: May 14, 2001

I hereby certify that the attached English Translation is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" Service on the date indicated above, and is addressed to:
Commissioner for Patents, Box PCT, Attn: EO/US,
Washington, DC 20231

Paula S. Dickey
Signature

USE OF A SUBSTANCE WHICH BINDS TO THE PERIPHERAL BENZODIAZEPINE RECEPTOR IN THE TREATMENT OF CUTANEOUS STRESS

The present invention relates to a composition for topical use containing a ligand for peripheral benzodiazepine receptors.

The invention relates to the use of a substance which binds specifically to the peripheral benzodiazepine receptor (PBR) for the manufacture of a composition for the prophylaxis or treatment of cutaneous stress.

The invention also relates to compositions containing these substances. These compositions may be cosmetic or pharmaceutical, and in particular topical dermatological compositions.

The term "cutaneous stress" means the various situations which may cause damage in particular to the epidermis, irrespective of the agent causing this damage. This agent may be inside and/or outside the body, for instance a chemical or free-radical agent or alternatively an external agent such as ultraviolet radiation.

The composition according to the invention is thus intended to prevent and combat skin irritation, dry patches, erythema, dysesthetic sensations, sensations of heating, pruritus of the skin and/or mucous membranes, and ageing, and may also be used in skin disorders such as, for example, psoriasis,

pruriginous diseases, herpes, photodermatitis, atopic dermatitis, contact dermatitis, lichens, prurigo, pruritus, insect bites, in fibrosis and other disorders of collagen maturation, in immunological disorders or in dermatological conditions such as eczema.

The PBR ligand, also referred to as "substance", contained in the composition may be a non-peptide compound, a peptide, a cell extract or tissue extract of animal or plant origin or a product obtained by fermenting a microorganism, for example fermenting a bacterium or fungus.

Many PBR ligands are disclosed in the literature. Examples which may be mentioned include Ro 5-4864 or chlorodiazepam, Ro 5-2807 or diazepam and PK 11195, or reference may be made to the article Peripheral Benzodiazepine Receptors, Ch. III, J.J. Bourguignon, Ed. E. Giesen - Crouse, Academic Press.

PBR is an 18-kd protein located on the outer membrane of the mitochondria of peripheral tissues. It consists of five transmembrane domains and of a carboxy-terminal portion directed towards the cytosol. Several functions are attributed to PBR depending on the nature of the tissue under consideration: regulation of steroidogenesis, biosynthesis of heme, cell differentiation and growth, control of mitochondrial respiration (Krueger KE, Biochimica and Biophysica Acta 1995, *1241*, 453-470). Although its precise function has not yet been fully elucidated,

several recent experimental data suggest that PBR might play a fundamental role in regulating the processes of programmed cell death and in protection against free radicals.

It has been shown that PBR is in fact closely associated at the mitochondrial level with apoptosis regulatory proteins such as Bcl2 which prevents rupture of the mitochondrial membrane potential, thus preventing the apoptosis induced in particular by the production of reactive oxygenated radicals (Marchetti P. *et al.*, J. Exp. Med. 1996, *184*, 1155-1160); (Marchetti P. *et al.*, J. Immunol. 1996, *157*, 4830-4836).

In the context of the present invention, the protective role of PBR against free radicals was directly observed on cells of hematopoietic origin for which a close correlation between the PBR density and the protection against free radicals was demonstrated. Furthermore, in this same study, it was demonstrated that the transfection of PBR into cells lacking this receptor gives protection against the damage caused by oxygenated species (Carayon P. *et al.*, Blood 1996, *87*, 3170-3178).

Several literature data suggest that PBR might play an important role in regulating apoptosis processes and in protecting cells against damage caused by free radicals.

Recent phylogenic studies reinforce this novel notion that PBR acts as an apoptosis modulator involved in antioxidant functions. The reason for this is that significant similarities exist between PBR and the protein CrtK of *Rhodobacter sphaeroides*, a photosynthetic bacterium. This bacterial protein which functions as a photosensitive oxygen detector, regulates the expression of the genes involved in photosynthesis in response to environmental changes in oxygen tension and in light intensity. The comparison between PBR and CrtK reveals 35% identity and a conservation of sequence between these two proteins which diverged in the phylogeny two billions years ago. This homology suggests a highly specialized and conserved function of PBR which appears to be similar to that of CrtK in the bacterium. Specifically, it has recently been demonstrated that mammalian PBR transfected into *Rhodobacter* CrtK mutants complements the oxygen-detecting function of CrTK. Thus, this study suggests a key role of PBR in the transduction of oxygen-dependant signals (Yeliseev AA., *et al.*, Proc. Natl. Acad. Sci. 1997, *94*, 5101-5106).

However, to date, no substance has ever been precisely indicated as a specific ligand for cutaneous PBR receptors, which is all the more reason why no topically active substance which binds specifically to the PBR receptors has ever been disclosed in the literature.

It has now been shown, in the context of the present invention, that PBR is abundantly expressed in the skin within the various cell compartments of which it is composed: keratinocytes, Langerhans cells, hair follicles and endothelial cells of the dermal vascular system. In the skin, the expression of PBR follows an increasing gradient from the basal layer to the horny layer. This noteworthy organization which favors the differentiated cells of the epidermis that are the most exposed to external stresses is undoubtedly of primordial physiological importance for protecting the most vulnerable areas of the epidermis. Subcellular studies performed by confocal microscopy indicate, as expected, a colocalization of PBR with Bcl2 in the mitochondrian. Histological studies on skin sections have revealed a surprising distribution of PBR (Figures 1 and 2).

Specifically, the expression of this receptor in the epidermis follows a gradient of increasing density from the basal layers to the most differentiated layers of keratinocytes. This highly organized spatial distribution which favors, in terms of density, the outermost and thus the most exposed cells of the epidermis, leads to the assumption that PBR in the skin might represent a natural protection system against free radicals generated by exposure to ultraviolet radiation. The concomitant observation that the distribution of the anti-apoptotic protein Bcl2

obeys a strictly inverse gradient suggests a compensatory role of PBR in preserving the cells that are most differentiated.

This set of data which suggest a protective function of PBR, more particularly in the epidermis, has led to the discovery of natural or synthetic ligands, showing that their interaction with PBR could be beneficial in various situations of cutaneous stress induced by chemical or free-radical agents or alternatively following an exposure to UV.

Thus, according to one of its aspects, the present invention relates to the use of a ligand which is specific for PBR, Ro 5-4864, in cutaneous stress. This ligand is a PBR agonist.

According to another aspect of the invention and on the basis of these observations, a screening directed toward finding natural PBR ligands was undertaken and made it possible to isolate several fractions capable of interacting with this receptor. The potentially protective effect of these natural ligands was then evaluated in various tests inducing a cutaneous stress and in particular in tests of cutaneous erythema induced by UV irradiation. Radical-scavenging properties and skin repair capacities were also investigated.

Biochemical and pharmacological tests were used to demonstrate the activity and advantage of the substances in various situations of cutaneous stress.

The tests performed with PBR were aimed at showing its potential involvement in regulating apoptotic processes and in preserving skin cells against various deleterious stress situations.

EXAMPLE 1

Immunohistological studies of cutaneous localization of PBR

A Western blot analysis made it possible to demonstrate the abundant presence of PBR on six different lines of human keratinocytes and on normal human skin (Figure 1), using specific anti-PBR antibodies Ac 8D7 (anti-PBR mouse mAb, isotype IgG1, Dussossoy *et al.*, Cytometry, 1996, 24:39-48). At the subcellular level, the analyses performed by confocal microscopy confirm a colocalization of PBR at the mitochondrial level in keratinocytes (Figure 2).

An immunohistological study performed on a normal human epidermal section using the same antibody reveals a very particular organization since the expression of PBR increases from the *stratum basale* to the *stratum corneum*. This receptor is thus abundantly present on the keratinocytes that are most differentiated, located directly under the *stratum corneum* (Figure 3).

EXAMPLE 2

Binding and specificity studies

The binding studies were performed on the keratinocyte line A-431 (human epidermoid carcinoma

(ATCC, CRL-1555)) by displacement of the reference ligand [^{3}H]-PK11195. Scatchard analysis indicates a single binding site, a density of about 470 000 receptors per cell and high affinity of the ligand (KD = 1.5 nM) (Figure 4). The specificity of the binding to the peripheral receptor borne by the keratinocytes is confirmed by the pharmacological studies which show a decreasing efficacy of the displacement of the reference peripheral ligand (PK 11195) by the following ligands:

Ro 5-4864 = ($IC_{50} \approx 25$ nM) > diazepam ($IC_{50} \approx 100$ nM) >>> clonazepam (inactive at 3 200 nM). It is recalled that this last compound is a ligand of the central receptor for benzodiazepines, diazepam is mixed and Ro 5-4864 is specific for PBR (Figure 5).

EXAMPLE 3

Involvement of PBR in protection against oxygenated radicals

Two types of experiment are described in Figure 6. The first consists in comparing different lines of lymphoid or myeloid origin as regards their ability to withstand the toxicity of oxygenated radicals in relation with their level of expression of PBR. The results indicate a very strong correlation between the number of PBR sites per cell and the resistance to the toxicity induced by H_2O_2. There is also a similar correlation when, this time, the level of expression of Bcl2, a protein known to protect cells

against oxidative damage, is considered. These data, combined with the fact that Bcl2 and PBR are proteins located on the outer mitochondrial membrane, suggest that they may have common functions in cell protection. Interestingly, although the expression of PBR follows a density gradient which increases from the basal layer to the limit of the horny layer, the literature data indicate an inverse phenomenon for the expression of Bcl2, suggesting that during the differentiation of keratinocytes, PBR may take over from Bcl2 as regards the functions of protection against free radicals.

In the second experiment, the possible role of PBR in protection against the toxicity of free radicals is reinforced by the demonstration of the better viability, in the presence of H_2O_2, of PBR+ transfected Jurkat cells in comparison with homologous PBR- cells.

EXAMPLE 4

The anti-apoptotic activity of the active agents was measured on human keratinocytes and on fibroblasts (ATCC) which were inoculated in 35 mm Petri dishes (5×10^5 cells/well) in DMEM (Dubelco's Mode Eagle Medium) supplemented with 10% fetal calf serum and left to proliferate to the point of confluence. This culture medium is then drawn off, the cells are rinsed and 0.1% fetal calf serum is added in the presence of a saline solution. Increasing concentrations of the substance to be studied are

added. Twenty four hours later, the apoptosis is measured with an ELISA (enzyme-linked immunosorbent assay) assay kit.

Keratinocytes were subjected to ultraviolet radiation of type B (UVB) at a dose of 250 J/m^2 for 16 hours (J. Invest. Dermatol. 1995, *104*: 922-927). In the presence of the PBR ligand Ro 5-4864, it was shown that the cell impairment processes induced by the irradiation are prevented in a ligand concentration range of between 10 nM and 10 µm.

EXAMPLE 5

The photoprotective effect of the ligand was evaluated by cutaneous application to albino guinea pigs.

The cutaneous topical route is used in order to reproduce the conditions of utilization in man.

Harley guinea pigs, from Charles River France, Saint Aubin les Elbeuf, 76410 Cléon, France, are used.

The animals were shaved and the hair on the right and left hind flanks was then plucked 24 hours before the start of the treatment.

The animals were irradiated immediately before the first treatment. The energy is checked before each irradiation performed on the right and left flanks, in the UVB spectrum at a dose of 4 000 mJ/cm^2.

The right flank of the animals was treated with 0.2 ml of ligand solution immediately after

irradiation and then 2 and 5 hours after irradiation. The left flank will not be treated.

A Xeron high pressure vapor lamp (IDEM 300) will produce the irradiation.

The local reactions are read before treatment and then 5 and 24 hours after irradiation.
Erythema and edema were evaluated as follows:
Erythema
0 no erythema; 1 very mild, barely perceptible erythema; 2 distinct, pale pink erythema; 3 distinct, bright red erythema; 4 particularly intense erythema
Edema
0 no edema; 1 very mild edema (barely visible); 2 mild edema (contours well defined and swelling apparent); 3 moderate edema (thickness of about 1 mm); 4 serious edema (thickness greater than 1 mm and area greater than the area of application).

Examples of natural ligands for the PBR receptor which are produced by fermentation are described below with their activity.

A screening carried out on microorganism extracts performed on solid or liquid medium made it possible to select three strains of microorganisms (microscopic fungi and bacteria).

The three strains selected after various studies performed to optimize the conditions for producing significant amounts of culture extracts having good activities in the test for measuring the

interaction with the PBR receptor, have the references SRL 4988, SRL 5186 and SRL 5189.

The above three strains were filed at the CNCM of the Institut Pasteur: date of 27 August 1999 with the respective serial numbers I-2305, I-2306 and I-2307.

The strain SRL 4988 classified as *Nocardia species*, isolated from a soil sample, has the following ecologico-physiological properties, determined after culturing for two weeks at 28°C on ISP2 medium: negative phototroph, chemo-organotroph, mesophile and negative halophile. It is immobile and has open, non-verticillate whorls.

The strain SRL 5186 classified as *Streptomyces species*, isolated from a soil sample, has the following ecologico-physiological properties, determined after culturing for two weeks at 28°C on ISP2 medium: negative phototroph, chemo-organotroph, mesophile and negative halophile. It is immobile and has flexible, biverticillate hyphae.

The strain SRL 5189 classified as *Actinosinnema species* has the following ecologico-physiological properties, determined after culturing for two weeks at 28°C on ISP2 medium: negative phototroph, chemo-organotroph, mesophile, negative halophile. It is immobile and has flexible, monoverticillar hyphae.

These strains, and also their productive mutants, thus constitute a further subject of the invention.

After culturing on nutrient agar medium and several successive subculturings which produce an abundant and pure culture, a storage batch 0 of the stock strain and then primary and secondary inoculation batches are prepared.

To do this, a spore suspension is prepared from a culture on nutrient agar medium in a Petri dish and from an uptake medium; this medium contains a cryoprotective agent to ensure good viability of the spores during the storage by freezing.

The spore suspension obtained is distributed into cryotubes which will be stored at -80°C: these tubes constitute batch 0.

By following the same protocol, but using a tube from batch 0, a primary inoculation batch is prepared. Next, again according to the same protocol, a secondary inoculation batch is prepared from a primary inoculation cryotube. Manufacture of the inoculation batches 0, 1 and 2 ensures long-lasting availability of the strain and thus of the desired activity. The culturing of these three strains for obtaining natural ligands of the PBR receptor may be carried out in a similar manner with the usual aerobic culture means, i.e. liquid media in fermenters of any volume with in-line monitoring of the pH and the aeration.

EXAMPLE A SRL 4988

As an example of culturing in conical flasks for the strain SRL 4988: a secondary inoculation tube is used to inoculate Petri dishes prepared with a medium for promoting actinomycetes sporulation according to the composition:

Glucose	20 g
Soyoptim (SIO)	10 g
$CaCO_3$ (OMYA)	3 g
Agar type E	20 g
Distilled water qs	1 l

The cultures are incubated in dishes for 5 days at 28°C. A spore suspension is then obtained by adding 10 ml of a liquid medium of the composition below to each Petri dish:

Glucose	30 g
Soyoptim (SIO)	10 g
Tryptone U.S.P. (Biokar)	4 g
Yeast extract (Difco)	8 g
NaCl	2.5 g
$CaCO_3$	5 g
Casein hydrolyzate	5 g
Soybean papain peptone	5 g

the pH of which is adjusted to 7.0 before sterilization.

5 ml of the spore suspensions are used to inoculate sterile 250 ml flasks, containing 50 ml of the same medium, which constitute the precultures, incubated in a warm chamber at 28°C on a shaker with shelves, or in an autonomous incubator, the rotation speeds in either of the systems being set at 210 rpm.

After shaking for two days, the preculture flasks are used to inoculate the actual culture flasks at a rate of 5 ml of preculture medium per 500 ml conical flask containing culture medium (100 ml) having the composition:

Glycerol	10 g
Soluble starch	30 g
Soyoptim	15 g
Tryptone	2 g
Yeast extract	5 g
$CaCO_3$	5 g
Trace element solution	10 ml
Water qs	1 l
pH 7	

Composition of the trace element solution used:

$FeSO_4 \cdot 7\ H_2O$	1.0 g
$MnSO_4 \cdot 4\ H_2O$	1.0 g
$CaCl_2 \cdot 2\ H_2O$	0.025 g
$CaCl_2 \cdot 2\ H_2O$	0.10 g
H_3BO_3	0.56 g
$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	0.002 g
$ZnSO_4 \cdot 7\ H_2O$	0.20 g
Water qs	1 l

Thus, in this specific case, five preculture flasks were used to inoculate 40 culture flasks with 100 ml of culture medium per 500 ml conical flask, which, after shaken culturing for 6 days at 28°C in a warm chamber on a rotary shaker set at 210 rpm, give 4 liters of bacterial suspension.

The 4 liters of fermentation broth are centrifuged several times, at a temperature of 4°C and under a regime of 13 500 rpm (i.e. 27 500 × g with the rotor used), in order to separate out the biomass, i.e. the pellet combining the cells from the culture supernatant consisting mainly of water from the nutrient medium used and containing in solution residues of components of the nutrient medium and also metabolites produced and excreted by the bacterial cells during the various phases of their growth.

The biomasses and supernatants are then frozen at -20°C.

EXTRACTION OF THE NATURAL LIGANDS OF THE PBR RECEPTOR

The 4 liters of thawed supernatant are placed in a 10 liter beaker. 400 g of Amberlite XAD 16 polystyrene-divinylbenzene resin (Rohm & Haas) are added to the solution. The suspension is shaken using a motor equipped with a paddle shaft, rotating at 20 rpm, for 15 hours. The solution is then filtered, the filtrate is removed and the drained resin is taken up in 1 liter of methanol. This mixture is stirred gently for 1 hour. The resin is again filtered off and retreated in an identical manner with 1 liter of methanol. During a third operation, the resin is retreated, this time with 1 liter of acetone. The drained resin is then removed and the 3 liters of combined organic solvent are evaporated to dryness in a rotary evaporator under vacuum.

The evaporation residue (17.7 g) is slurried in 50 ml of methanol, the suspension obtained is centrifuged at 3 000 rpm for 15 minutes and the settled supernatant obtained constitutes the culture supernatant extract.

This extract is tested in dilution for inhibition of the binding to the PBR receptor, and gives an activity evaluated at 1/200 (50% inhibition).

The combined biomasses (199 g) in a 2 liter beaker are treated, with stirring, with a mixture of 750 ml of methylene chloride and 750 ml of methanol. Stirring is continued for 15 hours at room temperature.

The suspension is then filtered and the clear solution obtained is concentrated under vacuum in a rotary evaporator. The evaporation residue (5.4 g) is then slurried in 50 ml of methanol and constitutes the biomass extract.

This extract is tested for inhibition of the binding of the PBR receptor, and gives an activity measured at 1/2200 (ID 50 = 2200^{-1}).

EXAMPLE B SRL 5186

With the same respective protocols and media:

- agar medium for the subculturings on Petri dishes
- liquid preculture medium
- liquid production medium,

14 × 500 ml conical flasks containing 100 ml of production medium, and inoculated to 5%, are incubated at 28°C in a warm chamber on a rotary shaker rotating at 210 rpm, for 6 days.

After centrifugation and storage of the production supernants and biomasses for one to two days in a freezer at -20°C, these products are thawed before proceeding with their extraction.

The biomasses (54.9 g) are treated in a beaker, with stirring, with a mixture of 250 ml of dichloromethane and 250 ml of methanol, for ten hours. The suspension is then filtered and the clear solution obtained is concentrated to dryness on a rotary evaporator.

The dry residue (1.4 g) is slurried in 17.5 ml of methanol and the suspension obtained is centrifuged at 3 000 rpm for 15 minutes. The centrifugation supernatant collected constitutes the biomass extract.

This extract, evaluated in dilution on the test for inhibition of binding to the PBR receptor, gives a 50% inhibition in the test at a dilution of 1/3750 (ID_{50} = 3 750^{-1}).

160 g of XAD 16 polystyrene-divinylbenzene resin (Rohm & Haas) are added to the 1 400 ml of thawed supernatant and the suspension is stirred for 15 hours. The resin is filtered off, the filtrate is removed and the resin is retreated with 200 ml of solution containing 25% methanol in water for 3 hours.

The resin is filtered off and this second filtrate is removed. The resin then undergoes three similar treatments, two with 200 ml of methanol and the last with 200 ml of acetone. These last three filtrates are combined in a round-bottomed flask and then concentrated under vacuum on a rotary evaporator. The dry residue obtained (2.2 g) is then slurried in 17.5 ml of methanol and the solution obtained constitutes the supernatant extract.

This extract, evaluated in dilution on the test for inhibition of the binding to the PBR receptor, gives a 50% inhibition at a dilution of 1/940 (ID_{50} = 940^{-1}).

EXAMPLE C SRL 5189

With the same respective protocols and media:

- agar medium for the subculturings on Petri dishes
- liquid preculture medium
- liquid production medium,

10 × 500 ml conical flasks containing 100 ml of production medium, inoculated to 5%, are incubated at 28°C in a warm chamber on a rotary shaker rotating at 210 rpm, for 8 days.

After centrifugation and storage of the production supernants and biomasses for one to two days in a freezer at -20°C, these products are thawed before proceeding with their extraction.

The biomasses (69.5 g) are treated in a beaker, with stirring, with a mixture of 150 ml of dichloromethane and 150 ml of methanol, for ten hours. The suspension is then filtered and the clear solution obtained is concentrated to dryness on a rotary evaporator. The dry residue (1.5 g) is slurried in 12.5 ml of methanol and the solution obtained is centrifuged at 3 000 rpm for 15 minutes. The centrifugation supernatant collected constitutes the biomass extract. This extract, evaluated in dilution on the test for inhibition of the binding to the PBR receptor, gives a 50% inhibition in the test at a dilution of 1/2600 (ID_{50} = 2 600^{-1}).

100 g of XAD 16 polystyrene-divinylbenzene resin (Rohm & Haas) are added to the 1 000 ml of thawed

supernatant and the suspension is stirred for 15 hours. The resin is filtered off, the filtrate is removed and the resin is retreated with 150 ml of a solution containing 25% methanol in water, for 3 hours. The resin is filtered off and this second filtrate is removed. The resin then undergoes three similar treatments, two with 150 ml of methanol and the last with 150 ml of acetone. These last three filtrates are combined in a round-bottomed flask and then concentrated under vacuum on a rotary evaporator. The dry residue obtained (1.7 g) is then slurried in 12.5 ml of methanol and the solution obtained constitutes the supernatant extract.

This extract, evaluated in dilution on the test for inhibition of the binding to the PBR receptor, gives a 50% inhibition at a dilution of 1/600 (ID_{50} = 500^{-1}).

In the compositions according to the invention, the substance which binds to PBR is preferably used in an amount ranging from 0.00001 to 20% by weight relative to the total weight of the composition and in particular in an amount ranging from 0.001% to 10% by weight relative to the total weight of the composition.

The compositions according to the invention may be in any presentation form normally used for topical application.

The amounts of the various constituents in the compositions according to the invention are those conventionally used in the fields under consideration and are appropriate for their presentation form.

For a topical application, the compositions of the invention comprise a medium which is compatible with the skin. These compositions may especially be in the form of aqueous, alcoholic or aqueous-alcoholic solutions, gels, water-in-oil or oil-in-water emulsions having the appearance of a cream or a gel, micro-emulsions or aerosols, or alternatively in the form of vesicular dispersions containing ionic and/or nonionic lipids. These presentation forms are prepared according to the usual methods of the fields under consideration.

These compositions for topical application may in particular constitute a cosmetic or dermatological protective, treatment or care composition for the face, for the neck, for the hands or for the body (for example day creams, night creams, antisun creams or oils or body milks), a make-up composition (for example a foundation) or an artificial tanning composition.

When the composition of the invention is an emulsion, the proportion of fatty substances it contains may range from 5% to 80% by weight and preferably from 5% to 50% by weight relative to the total weight of the composition. The fatty substances and emulsifiers used in the composition in emulsion

form are chosen from those conventionally used in cosmetics or dermatology.

As fatty substances which may be used in the invention, mention may be made of mineral oils (petroleum jelly), plant oils (liquid fraction of karite butter) and hydrogenated derivatives thereof, animal oils, synthetic oils (perhydrosqualene), silicone oils (polydimethylsiloxane) and fluoro oils. Other fatty substances which may also be mentioned included fatty alcohols (cetyl alcohol or stearyl alcohol), fatty acids (stearic acid) and waxes.

The emulsifiers may be present in the composition in a proportion ranging from 0.3% to 30% by weight and preferably from 0.5% to 30% by weight relative to the total weight of the composition.

In a known manner, the cosmetic or dermatological compositions of the invention may also contain adjuvants that are common in the corresponding fields, such as hydrophilic or lipophilic gelling agents, preserving agents, antioxidants, solvents, fragrances, fillers, screening agents and dyestuffs. Moreover, these compositions may contain hydrophilic or lipophilic active agents. The amounts of these various adjuvants or active agents are those conventionally used in cosmetics or dermatology, and, for example, from 0.01% to 20% of the total weight of the composition. Depending on their nature, these adjuvants or these active agents may be introduced into the fatty

phase, into the aqueous phase and/or into the lipid vesicles.

Among the active agents which the compositions of the invention may contain, mention may be made in particular of active agents which have an effect on treating wrinkles or fine lines, and in particular keratolytic active agents. The term "keratolytic" means an active agent which has desquamating, exfoliant or scrubbing properties, or an active agent capable of softening the horny layer.

Among these active agents with an effect on treating wrinkles and fine lines, which the compositions of the invention may contain, mention may be made in particular of hydroxy acids and retinoids.

The hydroxy acids may be, for example, α-hydroxy acids or β-hydroxy acids, which may be linear, branched or cyclic, and saturated or unsaturated. The hydrogen atoms of the carbon chain may also be substituted with halogens, halogenated, alkyl, acyl, acyloxy, alkoxycarbonyl or alkoxy radicals containing from 2 to 18 carbon atoms.

The hydroxy acids which may be used are, in particular, glycolic acid, lactic acid, malic acid, tartaric acid, citric acid, 2-hydroxyalkanoic acid, mandelic acid, salicylic acid and the alkyl derivatives thereof, for instance 5-n-octanoylsalicylic acid, 5-n-dodecanoylsalicylic acid, 5-n-decanoylsalicylic acid, 5-n-octylsalicylic acid, 5-n-heptyloxysalicylic

acid or 4-n-heptyloxysalicylic acid, and 2-hydroxy-3-methylbenzoic acid or alkoxylated derivatives thereof, for instance 2-hydroxy-3-methoxybenzoic acid.

The retinoids may be in particular retinoic acid and derivatives thereof, retinol (vitamin A) and esters thereof such as retinyl palmitate, retinyl acetate or retinyl propionate, and salts thereof.

These active agents may be used in particular in concentrations ranging from 0.0001% to 5% by weight relative to the total weight of the composition.

CLAIMS

1. Use of at least one substance which binds to the peripheral benzodiazepine receptor, or PBR, for the manufacture of a cosmetic, pharmacological or dermatological topical composition in the treatment of cutaneous stress.

2. Use of at least one substance which binds to PBR for the manufacture of a cosmetic and/or dermatological topical composition for reducing wrinkles, reducing solar erythema or protecting against free radicals.

3. Use according to either of Claims 1 and 2, characterized in that the substance which binds to PBR is a PBR agonist chosen from synthetic molecules, natural extraction substances and a substance obtained by fermentation.

4. Use according to any one of Claims 1 to 3, characterized in that the substance which binds to PBR is Ro 5-4864.

5. Use according to any one of Claims 1 to 3, characterized in that the substance which binds to PBR is a substance obtained by fermentation.

6. Use according to any one of Claims 1 to 5, characterized in that the substance is present in the cosmetic or dermatological composition in an amount ranging from 0.00001% to 20% by weight relative to the total weight of the composition.

7. Use according to any one of Claims 1 to 6, characterized in that the agonist substance is present in the cosmetic or dermatological composition in an amount ranging from 0.001% to 10% by weight relative to the total weight of the composition.

8. Use according to any one of Claims 1 to 7, characterized in that the composition also contains a hydroxy acid and/or a retinoid.

9. Use according to Claim 8, characterized in that the hydroxy acid is chosen from α-hydroxy acids and β-hydroxy acids, which may be linear, branched or cyclic, and saturated or unsaturated.

10. Use according to Claim 9, characterized in that the retinoid is chosen from the group comprising retinoic acid and derivatives thereof and retinol and esters thereof.

11. Cosmetic and/or dermatological topical composition, characterized in that it contains a substance which binds to PBR as active principle.

12. Strain *Nocardia species* SRL 4988 filed at the C.N.C.M. of the Institut Pasteur under No. I-2305 and its productive mutants.

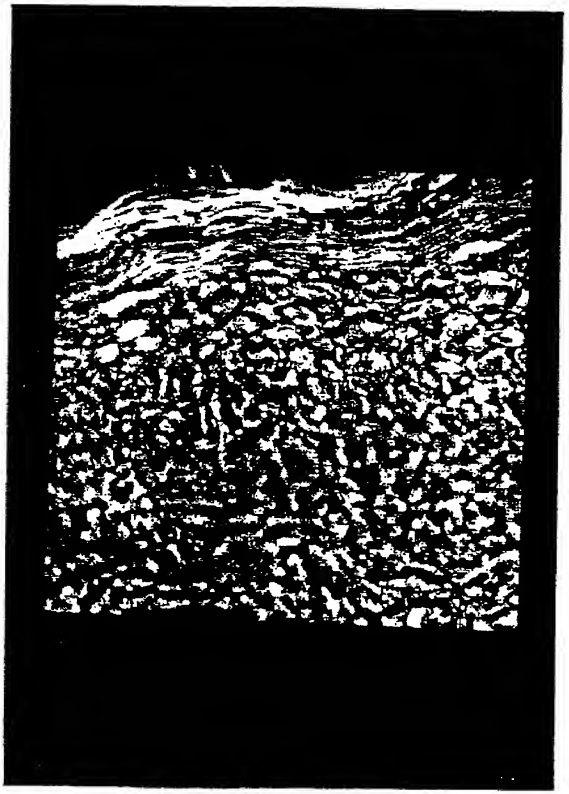
13. Strain *Streptomyces species* SRL 5186 filed at the C.N.C.M. of the Institut Pasteur under No. I-2306 and its productive mutants.

14. Strain *Actinosinnema species* SRL 5189 filed at the C.N.C.M. of the Institut Pasteur under No. I-2307 and its productive mutants.

15. Cosmetic and/or dermatological topical composition, characterized in that it contains a substance obtained by fermenting a strain according to Claims 12 to 14 as active substance.

Analysis by confocal microscopy using the antibody 8D7 of the mitochondrial localization of the PBR receptor on keratinocytes A431 (green coloration).

FIGURE 1



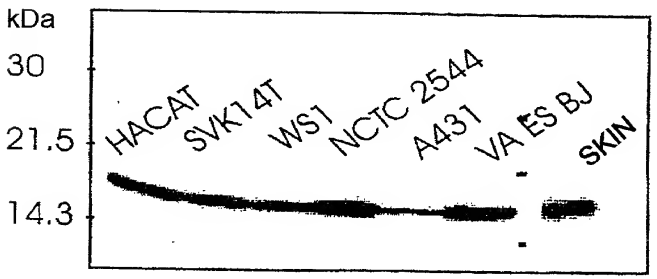
The immunohistological analysis performed on a section of normal human epidermis reveals an expression of PBR which increases from the *stratum basale* to the *stratum corneum* (red coloration).

FIGURE 2

Expression of PBR on keratinocyte lines

and on normal human skin lines:

Western blot analysis



[kDa] [SKIN]

8D7 antibody labeling (1 µg/ml final)

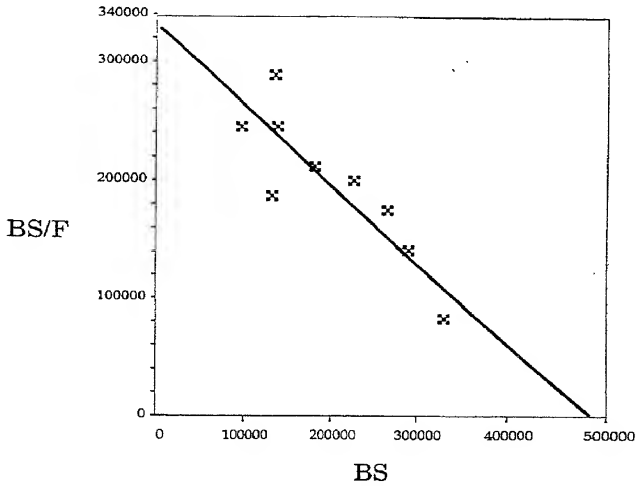
The deposits are normalized by assaying the total proteins of the lysate:

deposits for each line 30 µg

FIGURE 3

Scatchard study

on keratinocytes A431



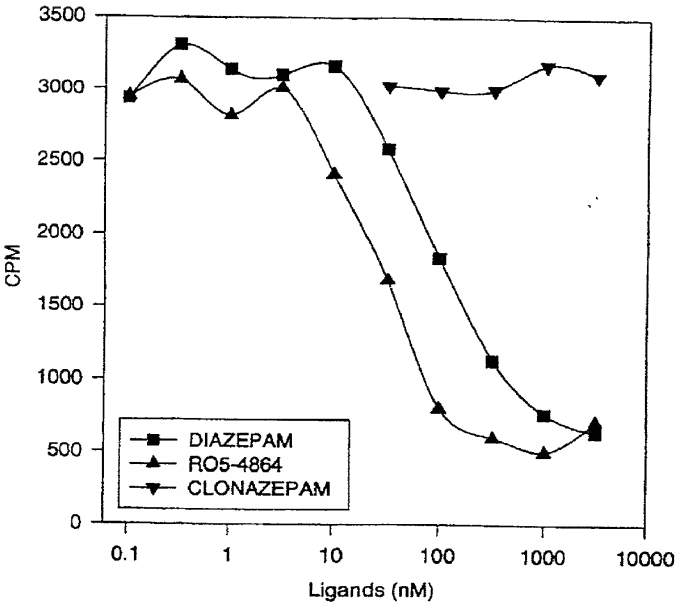
B max = 472 000 ± 68 000 receptors/cell

KD = 1.5 ± 0.3 mM

FIGURE 4

Pharmacological profile of ligands for PBR

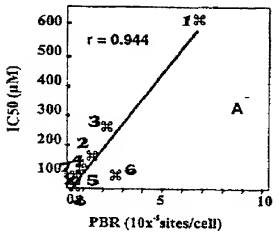
on keratinocytes A431



Curve of displacement of the reference ligand [3H]-PK11195 by Ro 5-4864 (peripheral ligand), clonazepam (central ligand) and diazepam (mixed ligand)

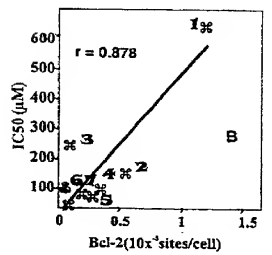
FIGURE 5

Involvement of PBR in the protection of hematopoietic cells against damage caused by oxygenated radicals

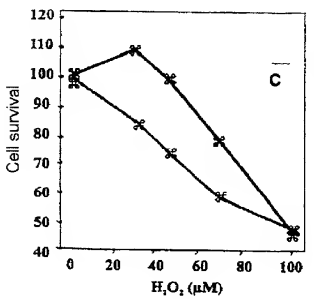


Correlation between the level of expression PBR [**A**] and of Bcl-2 [**B**] and of resistance to the toxicity of H_2O_2

1 = THP_1 2 = U937 3 = K562 IM9 5 = CEM 6 = NALM-6 7 = Jurkat 8 = RAJI

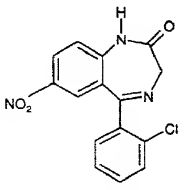


The H_2O_2 concentrations which induce 50% toxicity after incubation for 24 h [IC_{50}] are expressed as a function of the number of PBR or Bcl-2 sites.

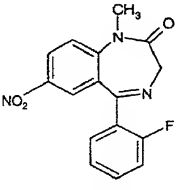


Viability of wild-type Jurkat cells ⌘ and of cells transfected with PBR ⌘ with respect to H_2O_2 toxicity after incubation for 24 h

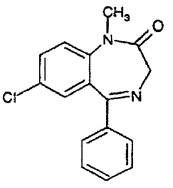
FIGURE 6



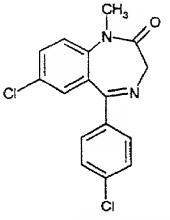
CLONAZEPAM



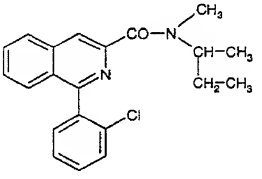
FLUNITRAZEPAM



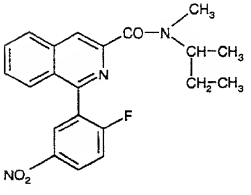
DIAZEPAM
Ro 5-2807



CHLORODIAZEPAM
Ro 5-4864



PK 11195



PK 14105

Main ligands for the central and peripheral benzodiazepine receptors

FIGURE 7

Attorney Docket No. 1087/PM

DECLARATION AND POWER OF ATTORNEY FOR UNITED STATES PATENT APPLICATION

__X__ Original _____ Supplemental _____ Substitute

As a below-named inventor, I hereby declare that:

My residence, citizenship and post office address are given below under my name.

I believe I am an original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

Use of a substance binding with the peripheral benzodiazepin receptor for treating skin stress

the specification of which

___ is attached hereto.

___ was filed on _____ as United States
Application Serial No. _____
and was amended on _____ (if applicable).

__X__ was filed on 10 November 1999 as PCT International
Application No. PCT/FR99/02761
and was amended under PCT Article 19 on _____ (if applicable).

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge my duty to disclose information of which I am aware which is material to the examination of this application in accordance with Section 1.56 of Title 37 of the Code of Federal Regulations.

I hereby claim foreign priority benefit under Section 119 (a) - (d) of Title 35 of the United States Code of any foreign application(s) for patent or inventor's certificate or of any PCT application(s) designating at least one country other than the United States identified below and also identify below any foreign application(s) for patent or inventor's certificate or any PCT application(s) designating at least one country other than the United States filed by me on the same subject matter and having a filing date before that of the application(s) from which priority is claimed:

			Priority Claimed	
Country	Number	Filing Date	Yes	No
France	98 14387	17 November 1998	X	

I hereby claim benefit under Section 120 of Title 35 of the United States Code of any United States application(s) or PCT application(s) designating the United States identified below and, insofar as the subject matter of each of the claims of this application is not disclosed in said prior application(s) in the manner provided by the first paragraph of Section 112 of Title 35 of the United States Code, I acknowledge my duty to disclose material information of which I am aware as defined in Section 1.56 of Title 37 of the Code of Federal Regulations which occurred between the filing date of the prior application(s) and the national or PCT filing date of this application:

Application Serial No.	Filing Date	Status

I hereby appoint Michael D. Alexander, Reg. No. 36,080; and Paul E. Dupont, Reg. No. 27,438, or any of them my attorneys or agents with full power of substitution and revocation to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

SEND CORRESPONDENCE TO:

Patent Department
Sanofi-Synthelabo Inc.
9 Great Valley Parkway
P.O. Box 3026
Malvern, PA 19355

DIRECT TELEPHONE CALLS TO:

MICHAEL D. ALEXANDER

Telephone No. (610) 889-8802

I hereby declare that all statements made herein and in the above-identified specification of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

100

Full name of first joint inventor: CASELLAS Pierre

Inventor's signature: _____ Date: 25/04/2001

Residence: 10 rue Carl Van Linné, FR-34090 MONTPELLIER

Citizenship: French FRX

200

Full name of second joint inventor: DEROCQ Jean-Marie

Inventor's signature: _____ Date: 18/04/2001

Residence: 6 rue des Clauzes, FR-34570 MURVIEL LES MONTPELLIER

Citizenship: French FRX